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NEW BOVINE AND HUMAN ACIDIC FIBROBLAST GROWTH FACTORS + USEFUL FOR STIMULATING DNA SYNTHESIS IN RESPONSIVE CELLS AND, FOR WOUND HEALING
ESP.

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(71) Applicant: MERCK & CO. INC.
126, East Lincoln Avenue P.O. Box 2000
Rahway New Jersey 07065-0900(US)

(72) Inventor: Linemeyer, David L.
526 Clark Street
Westfield New Jersey 07090(US)

(72) Inventor: Kelly, Linda J.
219 Bronx River Road Apt. 41
Yonkers New York 10704(US)

(72) Inventor: Thomas, Kenneth A., Jr.
245 Washington Avenue
Chatham New Jersey 07928(US)

(72) Inventor: Gimenez-Gallego, Guillermo
2652 Kennedy Boulevard
Jersey City New Jersey 07306(US)

(74) Representative: Hesketh, Alan, Dr.
European Patent Department Merck & Co., Inc. Terlings Park Eastwick Road
Harlow Essex, CM20 2QR(GB)

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(54) Cloning and expression of acidic fibroblast growth factor.

(57) Unique genes coding for the amino acid sequence of bovine acidic fibroblast growth factor (aFGF) and human aFGF are constructed. The bovine gene is derived from reverse translation of the aFGF amino acid sequence while the human gene is derived by specific point mutations of the bovine gene. Each gene construct is inserted into an expression vector which is used to transform an appropriate host. The transformed host cells produce recombinant aFGF (r-aFGF), human or bovine, which is purified and has an activity equivalent to the native protein.

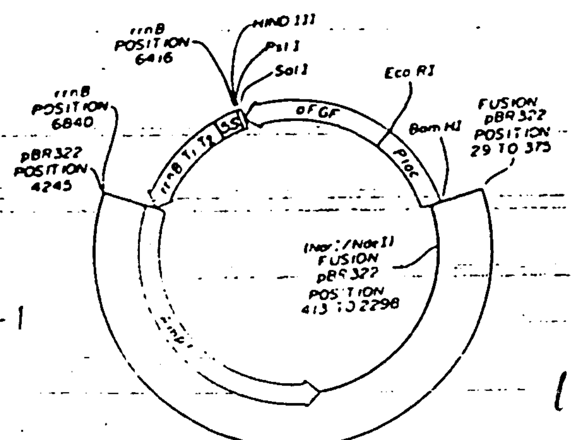

FIG-1

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TITLE OF THE INVENTION

CLONING AND EXPRESSION OF ACIDIC FIBROBLAST GROWTH FACTOR

BRIEF DESCRIPTION OF THE DRAWING

Figure I is a diagram of the pKK223-3 plasmid containing the gene for aFGF.

BACKGROUND OF THE INVENTION

Brain derived fibroblast mitogens were first described by Trowell et al., J. Exp. Biol. 16: 60-70 (1939) and Hoffman, Growth 4: 361-376 (1940). It was subsequently shown that pituitary extracts also had potent mitogenic activity for fibroblasts, Armelin, Proc. Natl. Acad. Sci USA 70: 2702-2706 (1973). Partial purification of both brain and pituitary fibroblast growth factor (FGF) revealed mitogenic activity against a variety of types of differentiated cells including vascular endothelial cells, Gospodarowicz et al., Natl. Cancer Inst. Monogr. 48: 109-130

(1978). It has recently been shown that FGF exists in two forms, acidic FGF (aFGF) and basic FGF (bFGF), and both forms have been identified in brain preparations, Thomas and Gimenez-Gallego, TIBS 11: 81-84 (1986). Numerous cell types respond to stimulation with either purified aFGF or bFGF to synthesize DNA and divide, including primary fibroblasts, vascular and corneal endothelial cells, chondrocytes, osteoblasts, myoblasts, smooth muscle and glial cells, Esch et al., Proc. Natl. Acad. Sci. USA 82: 6507-6511 (1985); Kuo et al., Fed. Proc. 44: 695 (1985).

Pure bovine brain-derived aFGF not only acts as a potent mitogen for vascular endothelial cells in culture but also induces blood vessel growth in vivo, Thomas et al., Proc. Natl. Acad. Sci. USA 82: 6409-6413 (1985). The fibroblast mitogenic activity of aFGF can also be utilized to promote wound healing, Thomas, U.S. Patent 4,444,760. The present invention provides a genetic construct and means of expression that allows the production of large amounts of pure aFGF that can be used therapeutically.

OBJECTS OF THE INVENTION

It is, accordingly, an object of the present invention to provide a nucleotide base sequence for both bovine aFGF and human aFGF from the amino acid sequences of the specific proteins. Another object is to produce genes coding for the specific aFGFs and incorporate the genes into appropriate cloning vectors. A further object is to transform an appropriate host with each of the recombinant vectors and to induce expression of the specific aFGF genes.

Another object is to isolate and purify biologically active bovine aFGF and human aFGF. These and other objects of the present invention will be apparent from the following description.

SUMMARY OF THE INVENTION

Unique genes coding for the amino acid sequence of bovine acidic fibroblast growth factor (aFGF) and human aFGF are constructed. The bovine gene is derived from reverse translation of the aFGF amino acid sequence while the human gene is derived by specific point mutations of the bovine gene. Each gene construct is inserted into an expression vector which is used to transform an appropriate host. The transformed host cells produce recombinant aFGF (r-aFGF), human or bovine, which is purified and has an activity equivalent to the native protein.

DETAILED DESCRIPTION

Acidic fibroblast growth factor exists in various microheterogeneous forms which are isolated from the various tissue sources and cell types known to contain aFGF. Microheterogeneous forms as used herein refers to a single gene product, that is a peptide produced from a single gene unit of DNA, which is structurally modified following translation. The structural modifications, however, do not result in any significant alterations of biological activity of the peptide. The modifications may take place either *in vivo* or during the isolation and purification process. *In vivo* modification results in but is not limited to proteolysis, glycosylation, phosphorylation or acetylation at the

N-terminus. Proteolysis may include exoproteolysis wherein one or more terminal amino acids are sequentially, enzymatically cleaved to produce a microheterogeneous form which has fewer amino acids than the original gene product. Endoproteolytic modification results from the action of endoproteases which cleave the peptide at specific locations within the amino acid sequence. Similar modifications can occur during the purification process which also results in production of micro- heterogeneous forms. The most common modification occuring during purification is proteolysis which is generally held to a minimum by the use of protease inhibitors. Under most conditions a mixture of microheterogeneous forms are present following purification of native aFGF. Native aFGF refers to aFGF isolated and purified from tissues or cells that contain aFGF.

The invention is contemplated to include all mammalian microheterogeneous forms of acidic fibroblast growth factor. The preferred embodiments include bovine and human microheterogeneous forms of aFGF. The most perferred microheterogeneous forms of bovine aFGF include a 154 amino acid form, a 140 amino acid form and a 134 amino acid form. The 140 amino acid form is shown in TABLE III and is the most preferred of the bovine species. The 154 amino acid form includes the following additional amino acids; Ala-Glu-Gly-Glu-Thr-Thr-Thr-Phe-Thr-Ala-Leu-Thr-Glu-Lys, with the carboxyl terminus Lys attached to the amino terminus Phe at the first position of the 140 amino acid form. The 134 amino acid form is identical to the 140 amino acid form except that the

first 6 amino acids of the amino terminus have been removed. When isolated the relative amounts of these microheterogeneous forms vary depending on the process used but all preparations contain at least a portion of each form.

Human aFGF exhibits a similar microheterogeneity to that of bovine aFGF. The most preferred microheterogeneous forms of human aFGF include a 154 amino acid form, a 140 amino acid form and a 139 amino acid form. The human 140 amino acid form differs from the bovine form by eleven amino acids, as shown in TABLE V. The 154 amino acid form contains the exact sequence of the human 140 amino acid form plus the 14 additional amino acids associated with the bovine 154 amino acid form, with one exception. The amino acid at the fifth position of the N-terminus or at the -10 position as determined from the 140 amino acid Phe n-terminus in the human form is isoleucine and is substituted for the threonine in the bovine form. The additional 14 amino acid human N-terminal sequence is; Ala-Glu-Gly-Glu-Ile-Thr-Thr-Phe-Thr-Ala-Lue-Thr-Glu-Lys. A third form of human aFGF contains 139 amino acids and is equivalent to the human 140 amino acid form with the amino terminus phenylalanine removed. The amino terminus asparagine residue may be deamidated to aspartic acid in the 139 amino acid form of human aFGF. The 140 and 139 amino acid forms are the most preferred forms of the human microheterogeneous forms.

Mammalian r-aFGF is produced by cloning the natural gene from either the genomic DNA or cDNA, or by construction of a gene for one of the

microheterogeneous forms of the protein based on the known amino acid sequences of these microheterogeneous forms of aFGF from mammalian species including man. Genomic DNA is extracted from mammalian brain or pituitary cells and prepared for cloning by either random fragmentation of high-molecular-weight DNA following the technique of Maniatis et al., Cell 15: 687-701 (1978) or by cleavage with a restriction enzyme by the method of Smithies et al., Science 202: 1284-1289 (1978). The genomic DNA is then incorporated into an appropriate cloning vector, generally E. coli lambda phage, see Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

To obtain cDNA for aFGF, poly (A)-containing RNA is extracted from cells that express aFGF by the method of Aviv and Leder, Proc. Natl. Acad. Sci. 69: 1408-1412 (1972). The cDNA is prepared using reverse transcriptase and DNA polymerase using standard techniques, as described in Maniatis et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982). The cDNA is tailed and cloned into an appropriate vector, usually pBR322, by a technique similar to that of Wensink, et al., Cell 3: 315-325 (1974).

The clonal genomic DNA or cDNA libraries are screened to identify the clones containing aFGF sequences by hybridization with an oligonucleotide probe. The sequence of the oligonucleotide hybridization probe is based on the determined amino acid sequence of aFGF. Maniatis et al. supra, Anderson and Kingston, Proc. Natl. Acad. Sci. USA

80:6838-6842 (1983) and Suggs et al., Proc. Natl. Acad. Sci. USA 78:6613-6617 (1981) describe various procedures for screening genomic and cDNA clones.

The preferred procedure for obtaining a gene for mammalian aFGF is to synthesize the gene. The gene may be synthesized based on the amino acid sequence of a microheterogeneous form of aFGF obtained from any mammal including man. The preferred method is to use the bovine amino acid sequence for aFGF and chemically point mutate the base sequence to produce the genes for other species. The amino acid sequences for bovine and human aFGF are disclosed in U.S. Patent Application Serial No. 868,473 filed May 30, 1986 which is a continuation-in-part of U.S. Patent Application Serial No. 774,359 filed September 12, 1985 which is a continuation-in-part of U.S. Patent Application Serial No. 685,923, filed December 24, 1984 (now abandoned).

The synthetic genes are based on the determined bovine amino acid sequence subsequently described by Gimenez-Gallego et al., Science 230: 1385-1388 (1985) and the human amino acid sequence as described by Gimenez-Gallego et al. Biochem. Biophys. Res. Comm., 138: 611-617 (1986). The unique nucleotide sequence of the 140 amino acid form of bovine aFGF is derived from reverse translation of the amino acid sequence by a technique similar to that of Itakura et al., Science 198: 1056-1063 (1977). The various novel nucleotide sequences corresponding to the native amino acid sequence of bovine aFGF are shown in the following table:

TABLE I

				5					10					15					20
Phe	Asn	Leu	Pro	Leu	Gly	Asn	Tyr	Lys	Lys	Pro	Lys	Leu	Leu	Tyr	Cys	Ser	Asn	Gly	Gly
TTQ	AAQ	CTN	CCN	CTN	GGN	AAQ	TAQ	AAP	AAP	CCN	AAP	CTN	CTN	TAQ	TGQ	TCN	AAQ	GGN	GGN
		TTP		TTP								TTP	TTP			AGQ			

				25					30					35					40
Tyr	Phe	Leu	Arg	Ile	Leu	Pro	Asp	Gly	Thr	Val	Asp	Gly	Thr	Lys	Asp	Arg	Ser	Asp	Gln
TAQ	TTQ	CTN	CGN	ATQ	CTN	CCN	GAQ	GGN	ACN	GTN	GAQ	GGN	ACN	AAP	GAQ	CGN	TCN	GAQ	CAP
		TTP	AGP	ATA	TTP											AGP	AGQ		

				45					50					55					60
His	Ile	Gln	Leu	Gln	Leu	Cys	Ala	Glu	Ser	Ile	Gly	Glu	Val	Tyr	Ile	Lys	Ser	Thr	Glu
CAQ	ATQ	CAP	CTN	CAP	CTN	TGQ	GCN	GAP	TCN	ATQ	GGN	GAP	GTN	TAQ	ATQ	AAP	TCN	ACN	GAP
	ATA		TTP		TTP				AGQ	ATA					ATA		AGQ		

				65					70					75					80
Thr	Gly	Gln	Phe	Leu	Ala	Met	Asp	Thr	Asp	Gly	Leu	Leu	Tyr	Gly	Ser	Gln	Thr	Pro	Asn
ACN	GGN	CAP	TTQ	CTN	GCN	ATG	GAQ	ACN	GAQ	GGN	CTN	CTN	TAQ	GGN	TCN	CAP	ACN	CCN	AAQ
				TTP							TTP	TTP			AGQ				

				85					90					95					100
Glu	Glu	Cys	Leu	Phe	Leu	Glu	Arg	Leu	Glu	Glu	Asn	His	Tyr	Asn	Thr	Tyr	Ile	Ser	Lys
GAP	GAP	TGQ	CTN	TTQ	CTN	GAP	CGN	CTN	GAP	GAP	AAQ	CAQ	TAQ	AAQ	ACN	TAQ	ATQ	TCN	AAP
			TTP		TTP		AGP	TTP									ATA	AGQ	
				105					110					115					120
Lys	His	Ala	Glu	Lys	His	Trp	Phe	Val	Gly	Leu	Lys	Lys	Asn	Gly	Arg	Ser	Lys	Leu	Gly
AAP	CAQ	GCN	GAP	AAP	CAQ	TGG	TTQ	GTN	GGN	CTN	AAP	AAP	AAQ	GGN	CGN	TCN	AAP	CTN	GGN
										TTP					AGP	AGQ		TTP	
				125					130					135					140
Pro	Arg	Thr	His	Phe	Gly	Gln	Lys	Ala	Ile	Leu	Phe	Leu	Pro	Leu	Pro	Val	Ser	Ser	Asp
CCN	CGN	ACN	CAQ	TTQ	GGN	CAP	AAP	GCN	ATQ	CTN	TTQ	CTN	CCN	CTN	CCN	GTN	TCN	TCN	GAQ
	AGP								ATA	TTP		TTP		TTP			AGQ	AGQ	

Where Q = C or T,
P = A or G, and
N = A, T, C, or G

The nucleotide sequence of the present invention incorporates the following characteristics: codons preferred by Escherichia coli and mammalian cells where possible, elimination of sequences with multiple complementarities, incorporation of unique restriction sites throughout the gene, terminal restriction enzyme sticky ends for ease of inserting the gene into plasmids, a centrally located unique restriction site to allow assembly of the gene in two halves, preferably an N-terminal methionine codon for a translational start site, and tandem translational stop codons.

While the following description and examples illustrate the present invention with respect to a particular nucleotide sequence for bovine aFGF, it is to be understood that the present invention could include any of the permutations listed in Table I. The following table contains the preferred nucleotide sequence:

TABLE II

TTCAATCTGCCCACTGGGTAATTACAAAAGCCAAAGCTTCTTTACTGCTCTAACGGTGGT	60
TACTTTCTCCGCATCCTGCCAGATGGTACCGTGGACGGCACCAAAGATCGTTCTGATCAA	120
CATATTCAACTGCAGCTGTGCGCCGAATCTATCGGTGAAGTTTACATCAAATCTACCGAA	180
ACTGGTCAATTCCTTGCCATGGACACTGATGGCCTGCTGTACGGATCCCAGACCCCAAAC	240
GAGGAGTGCCTTTTCCTGGAGCGCCTGGAGGAAAACCATTACAACACCTACATCTCTAAA	300
AAGCATGCTGAGAAACATTGGTTCGTAGGCCTTAAGAAAAATGGCCGCTCTAAACTGGGC	360
CCTCGTACTCACTTTGGTCAAAAAGCTATCCTGTTCCTGCCACTGCCAGTGAGCTCTGAC	420

The gene is constructed with a leader portion containing a single restriction enzyme cleavage site and an N-terminal methionine codon for a translational start site. The gene also contains a tail containing tandem translational stop codons and two restriction enzyme cleavage sites. The complementary characteristic of DNA allows a choice of base sequences which in turn allows for the incorporation of unique restriction enzyme cleavage sites throughout the gene. The preferred gene base sequence with the location of the restriction enzyme cleavage sites is shown in the following table:

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TABLE III

```
 1        10                  20                  30
 MetPheAsnLeuProLeuGlyAsnTyrLysLysProLysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThrValAspGlyThrLysAspArgSer

          [1]                              [3]
1     20        40          60          80        100         120
AATTCATGTTCAATCTGCCACTGGGTAATTACAAAAAGCCAAAGCTTCTTTACTGCTCTAACGGTGGTTACTTTCTCCGCATCCTGCCAGATGGTACCGTGGACGGCACCAAAGATCGTTCT
    GTACAAGTTAGACGGTGACCCATTAATGTTTTTCGGTTTCGAAGAAATGACGAGATTGCCACCAATGAAAGAGGCGTAGGACGGTCTACCATGGCACCTGCCGTGGTTTCTAGCAAGA
          [2]                              [4]
(EcoRI)              HindIII                          KpnI

 40                  50                  60                  70
 AspGlnHisIleGlnLeuGlnLeuCysAlaGluSerIleGlyGluValTyrIleLysSerThrGluThrGlyGlnPheLeuAlaMetAspThrAspGlyLeuLeuTyrGlySerGlnThr

          [5]                              [7]
      140       160          180         200        220          240
GATCAACATATTCAACTGCAGCTGTGCGCCGAATCTATCGGTGAAGTTTACATCAAATCTACCGAAACTGGTCAATTCCTTGCCATGGACACTGATGGCCTGCTGTACGGATCCCAGACC
CTAGTTGTATAAGTTGACGTCGACACGCGGCTTAGATAGCCACTTCAAATGTAGTTTAGATGGCTTTGACCAGTTAAGGAACGGTACCTGTGACTACCGGACGACATGCCTAGGGTCTGG
          [6]                              [8]
BclI      PstIPvuII  HinfI                    NcoI                BamHI
```

TABLE III (Cont'd)

```
80                                 90                         100                              110
ProAsnGluGluCysLeuPheLeuGluArgLeuGluGluAsnHisTyrAsnThrTyrIleSerLysLysHisAlaGluLysHisTrpPheValGlyLeuLysLysAsnGlyArgSerLys

     [9]                                                           [11]
         260                 280                    300                  320                340                 360
CCAAACGAGGAGTGCCTTTTCCTGGAGCGCCTGGAGGAAAACATTACAACACCTACATCTCTAAAAAGCATGCTGAGAAACATTGGTTCGTAGGCCTTAAGAAAAATGGCCGCTCTAAA
GGTTTGCTCCTCACGGAAAAGGACCTCGCGGACCTCCTTTTGGTAATGTTGTGGATGTAGAGATTTTTCGTACGACTCTTTGTAACCAAGCATCCGGAATTCTTTTTACCGGCGAGATTT
            [10]                                                         [12]
                 HaeII                                          SphI                 StuI

120                        130                        140
LeuGlyProArgThrHisPheGlyGlnLysAlaIleLeuPheLeuProLeuProValSerSerAsp

 [13]                                          [15]
           380                  400                    420                 440            440
CTGGGCCCTCGTACTCACTTTGGTCAAAAAGCTATCCTGTTCCTGCCACTGCCAGTGAGCTCTGACTAATAGATATCG
GACCCGGGAGCATGAGTGAAACCAGTTTTTCGATAGGACAAGGACGGTGACGGTCACTCGAGACTGATTATCTATAGCAGCT
         [14]                                    [16]
 ApaI                                                 SacI          EcoRV (SalI)
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The gene sequence for each strand of the double-stranded molecule is randomly divided into 8 nucleotide sequences. The oligonucleotides are constructed with overlapping ends to allow the formation of the double-stranded DNA. The following table contains one of a multitude of oligonucleotide arrangements that is used to produce the bovine aFGF gene.

TABLE IV

OLIGO-1 10 20 30 40 50 58

5' AATTCATGTT CAATCTGCCA CTGGGTAATT ACAAAAAGCC AAAGCTTCTT TACTGCTC 3'

OLIGO-2 10 20 30 40 45

5' AGAAGCTTTG GCTTTTTGTA ATTACCCAGT GGCAGATTGA ACATG 3'

OLIGO-3 10 20 30 40 50 60

5' TAACGGTGGT TACTTTCTCC GCATCCTGCC AGATGGTACC GTGGACGGCA CCAAAGATCG 3'

OLIGO-4 10 20 30 40 50 59

5' TGCCGTCCAC GGTACCATCT GGCAGGATGC GGAGAAAGTA ACCACCGTTA GAGCAGTAA 3'

OLIGO-5 10 20 30 40 46

5' TTCTGATCAA CATATTCAAC TGCAGCTGTG CGCCGAATCT ATCGGT 3'

OLIGO-6 10 20 30 40 50 60 65

5' GTAAACTTCA CCGATAGATT CGGCGCACAG CTGCAGTTGA ATATGTTGAT CAGAACGATC TTTGG 3'

OLIGO-7 10 20 30 40 50 60 67

5' GAAGTTTACA TCAAATCTAC CGAAACTGGT CAATTCCTTG CCATGGACAC TGATGGCCTG CTGTACG 3'

OLIGO-8 10 20 30 40 50 60 62

5' GATCCGTACA GCAGGCCATC AGTGTCCATG GCAAGGAATT GACCAGTTTC GGTAGATTTG AT 3'

OLIGO-9 10 20 30 40 50 52

5' GATCCCAGAC CCCAAACGAG GAGTGCCTTT TCCTGGAGCG CCTGGAGGAA AA 3'

OLIGO-10 10 20 30 40 50 58

5' GTTGTAATGG TTTTCCTCCA GGCGCTCCAG GAAAAGGCAC TCCTCGTTTG GGGTCTGG 3'

OLIGO-11 10 20 30 40 48

5' CCATTACAAC ACCTACATCT CTAAAAAGCA TGCTGAGAAA CATTGGTT 3'

OLIGO-12 10 20 30 40 46

5' GGCCTACGAA CCAATGTTTC TCAGCATGCT TTTTAGAGAT GTAGGT 3'

OLIGO-13 10 20 30 40 50 53

5' CGTAGGCCTT AAGAAAAATG GCCGCTCTAA ACTGGGCCCT CGTACTCACT TTG 3'

OLIGO-14 10 20 39 40 50 55

5' GCTTTTTGAC CAAAGTGAGT ACGAGGGCCC AGTTTAGAGC GGCCATTTTT CTTAA 3'

OLIGO-15 10 20 30 40 50 56

5' GTCAAAAAGC TATCCTGTTC CTGCCACTGC CAGTGAGCTC TGACTAATAG ATATCG 3'

OLIGO-16 10 20 30 40 50

5' TCGACGATAT CTATTAGTCA GAGCTCACTG GCAGTGGCAG GAACAGGATA 3'

The oligonucleotides illustrated in Table IV are presented merely as an example of oligonucleotide subunits and should not be construed as limiting thereto. The composite base sequence showing the overlap and arrangement of the oligonucleotides is illustrated in Table III.

The bovine gene is assembled in 2 steps: first, the half corresponding to the N-terminal portion of the protein; and second, the C-terminal half. Generally, the oligonucleotides are kinased with T4 polynucleotide kinase in the presence of either ATP or ^{32}P-labelled ATP. In the first reaction of each step the oligonucleotides which make up one strand of the gene are kinased with the exception of the most 5' oligonucleotide. In the second reaction the oligonucleotides which make up the second strand are kinased, with the exception of the most 5' oligonucleotide. When kinased oligonucleotides are used, about 1 pmole of the ^{32}P-labelled oligonucleotide is added for later identification of the products. Annealing is carried out in an appropriate buffer, such as one containing but not limited to about 60 mM TRIS, about pH 7.6, about 5 mM dithiothereitol (DTT), about 10 mM $MgCl_2$, and about 30 µM ATP at about 90°C for about 4 minutes followed by a rapid transfer to about 60°C and a slow cooling to about 30°C. Ligation is carried out in an appropriate buffer, such as one containing, but not limited to, about 60 mM TRIS, about pH 7.6, about 10 mM DTT, about 10 mM $MgCl_2$, about 1 mM ATP, and about 0.03 units T4 DNA ligase at about 20°C for about 1 and 1/2 hour.

The ligated oligonucleotides are purified by polyacrylamide gel electrophoresis following ethanol precipitation. The oligonucleotides are redissolved in a buffer containing about 20 µl of about 80% formamide, about 50 mM TRIS-borate, about pH 8.3, about 1 mM ethylenediaminetetraacetic acid (EDTA), about 0.1% (w/v) xylene cyanol, and about 0.1% (w/v) bromophenol blue. Each sample is heated at about 90°C for about 3 minutes and electrophoresed in about a 10% urea-polyacrylamide gel at about 75 watts for about 5 hours. The 231 base N-terminal bands are removed, combined and eluted at about 4°C in about 0.5 M ammonium acetate containing about 1mM EDTA at about pH 8. The 209 base C-terminal bands are treated in the same manner.

The synthetic gene sequences coding for either the N-terminal or the C-terminal portions of the aFGF are incorporated into the pBR322 plasmid. It is especially desired and intended that there be included within the scope of this invention, the use of other plasmids into which the aFGF gene can be incorporated and which will allow the expression of the aFGF gene. Reannealed oligonucleotides, about 300 fmole and about 100 fmole of the recovered 231 base pair N-terminus are each ligated to about 100 fmole of agarose gel purified about 3.9 kilo base (kb) EcoRI-BamHI pBR322 for the N-terminus. The 209 bp C-terminus is constructed in the same manner using BamHI-SalI pBR322. Ligation is carried out in a buffer containing about 25 mM TRIS, about pH 7.8, about 1 mM DTT, about 10 mM $MgCl_2$, about 0.4 mM ATP, with about 1 unit of T4 DNA ligase for about 1

hour at about 20°C. Each half-gene ligated vector is used to transform competent bacterial cells, such as E. coli RR1 (Bethesda Research Laboratories, BRL) following suppliers procedures. The transformed cells are selected for growth in ampicillin and screened for the presence of either the 231 base pair (bp) EcoRI-BamHI insert or the 209 bp BamHI-SalI insert by restriction analysis of mini-lysate plasmid preparations.

The DNA sequence of clones containing the appropriate sized inserts is determined using Maxam and Gilbert, Proc. Natl. Acad. Sci. USA 74: 560-564 (1977) chemical DNA sequence techniques.

The final full-length aFGF synthetic gene was cloned by cleaving the N-terminal half clone with restriction enzymes BamHI and SalI, treating with alkaline phosphatase and ligating this to the gel purified 209 bp BamHI-SalI insert of the C-terminal half clone. This ligated material was used to transform competent RR1 cells as before.

Expression of the synthetic aFGF gene is accomplished by a number of different promoter-expression systems. It is desired and intended that there be included within the scope of this invention, the use of other promoter-expression systems for the expression of the intact aFGF gene. The preferred construct uses the E. coli tac promoter, a hybrid between regions of the trp promoter and the lac promoter as described by deBoer et al., Proc. Nat. Acad. Sci. USA 80: 21-25 (1983). Plasmid pKK 223-3 (Pharmacia) which contains the tac promoter and rrnB rRNA transcription terminator was modified to remove

the pBR322-derived SalI restriction enzyme site. The rrnB rRNA terminator has been shown to allow expression by strong promoters, Gentz *et al*., Proc. Natl. Acad. Sci. USA 78: 4936-4940 (1981); Brosius, Gene 27: 161-172 (1984).

The pKK223-3 plasmid DNA is cleaved with restriction enzymes to produce a 2.7 kb DNA fragment to generate clone pKK 2.7. The synthetic aFGF gene is cleaved from its pBR322 vector and transferred to the pKK 2.7 plasmid after restricting pKK 2.7 with EcoRI and SalI. The resulting recombinant, shown in figure 1, is transformed into *E. coli* JM105 (Pharmacia) or DH5 (BRL) cells and expressed.

Site specific mutagenesis is an efficient way to convert the amino acid sequence of one mammalian species of aFGF to the aFGF amino acid sequence of another species. The following description relates to the site specific mutagenic conversion of bovine aFGF, 140 amino acid form, to human aFGF, it is to be understood, however, that the process can be used to convert any mammalian species aFGF to that of any other species. The only limitation on the conversion is that the amino acid sequences of both aFGFs must be known. The following table lists the amino acids which must be substituted and the location on the bovine aFGF amino acid map, Table III, at which the substitutions are made:

TABLE V

Amino Acid Location	Substituted Amino Acids Human aFGF	for Bovine aFGF
5	Pro	Leu
21	His	Tyr
35	Arg	Lys
47	Ser	Cys
51	Val	Ile
64	Tyr	Phe
106	Asn	His
116	Ser	Arg
117	Cys	Ser
119	Arg	Leu
125	Tyr	Phe

As with the bovine gene sequence eight oligonucleotides representing the human gene sequence are constructed by the same procedure as that used for the bovine oligonucleotides. The following table contains one of a multitude of oligonucleotide arrangements that is used to produce the human aFGF gene.

TABLE VI

OLIGO-1
5' CTGCCACCGGGTAATTAC 3'

OLIGO-2
5' CGGTGGTCACTTTCTCCG 3'

OLIGO-3
5' CGGCACCAGAGATCGTTC 3'

OLIGO-4
5' GCAGCTGTCCGCCGAATCTGTCGGTGAAG 3'

OLIGO-5
5' CTGGTCAATACCTTGCCATGG 3'

OLIGO-6
5' GCTGAGAAAAATTGGTTCG 3'

OLIGO-7
5' GGCCGCGTTTACAGCTGCCATTTTTCTTAAGG 3'

OLIGO-8
5' CGTACTCACTATGGCCAAAAAGCTATCC 3'

The cloned synthetic bovine gene for aFGF is converted to a human synthetic gene for aFGF by a series of directed point mutations. Oligonucleotide-directed mutagenesis of the cloned gene allows the alteration of the base sequence of bovine aFGF so that the resulting amino acid sequence contains the substituted amino acids shown in Table V and is human aFGF. A deletion is made in the bovine gene to remove the amino terminal phenylalanine for the production of the human 139 amino acid microheterogeneous form of aFGF. A point mutation is carried out to replace the second position asparagine with aspartic acid. Alternatively, the asparagine is deamidated to aspartic acid. The methods for carrying out these procedures are described below or are known in the art. The oligonucleotide-directed mutagenesis is carried out using standard procedures known to the art, Zoller and Smith, Methods in Enzymology, 100: 468-500 (1983); Norris et al., Nucleic Acids Research, 11: 5103-5112 (1983); and Zoller and Smith, DNA, 3: 479-488 (1984). The point mutations carried out by the standardized oligonucleotide-directed mutagenesis are shown in the following, Table VII. The location of the base mutagenesis can be seen in Table III. The point mutations are presented merely as an example of changes which will result in the human aFGF gene and should not be construed as limiting thereto.

TABLE VII

Base Location	Substituted Base Human aFGF	for Bovine aFGF	Corresponding Human Amino Acid
22	C	T	Pro
69	C	T	His
112	G	A	Arg
148	C	G	Ser
159	G	A	Val
199	A	T	Tyr
324	A	C	Asn
354	A	C	Ser
358	G	C	Cys
364	G	T	Arg
365	C	G	Arg
382	A	T	Tyr

The expression clones are grown at about 37°C in an appropriate growth medium, which consists of about 1% tryptone, about 0.5% yeast extract, about 0.5% NaCl, about 0.4% glucose and about 50 μg/ml ampicillin. When the optical density at 550 nm reaches about 0.5, isopropyl-ß-D-thiogalactopyranoside (IPTG) may be added to give a final concentration of about 1 mM and growth is continued at about 37°C for about 3 hours. The cells from 1 liter of culture medium are harvested by centrifugation and resuspended in a disruption buffer containing about 10 mM sodium phosphate at about pH 7.2, about 5 mM EDTA, about 10.6 μg/ml N-p-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), about 34.3 μg/ml pepstatin A, about 87 μg/ml phenylmethylsulfonyl

fluoride (PMSF), about 15 μg/ml bovine pancreatic trypsin inhibitor (BPTI), and about 25.2 μg/ml leupeptin. The cells are either immediately disrupted or frozen and stored at -70°C and disrupted immediately after thawing by about three passages through a French pressure cell at about 12,000 psi at about 4°C. The supernatant fluid is collected by centrifugation.

The recombinant aFGF is purified to homogeneity by a unique two-step chromatographic procedure employing a combination of heparin-Sepharose affinity chromatography followed by reversed-phase high performance liquid chromatography (HPLC). The crude r-aFGF is loaded onto a heparin-Sepharose column in a dilute buffer such as about 10 mM phosphate or Tris, about pH 6 to 8, which is subsequently washed with a low concentration of salt, such as about 0.8 M NaCl, until the absorbance at 280 nm drops to about background. The r-aFGF is eluted with a buffered high salt concentration solution such as about 10 mM sodium phosphate or Tris, about pH 6 to 8, containing about 1.5 M NaCl. The eluate is then purified by reversed-phase HPLC on a resin consisting of covalently linked alkyl silane chains with alkyl groups having from 3 to 18 carbon atoms, preferably 4 carbon atoms. The r-aFGF is directly applied to the HPLC column equilibrated in a dilute acid such as about 10 mM trifluoroacetic acid, acetic acid or phosphoric acid and eluted with a linear gradient of organic solvent such as acetonitrile or ethanol. Bovine brain-derived aFGF was previously described to bind to both heparin-Sepharose by Maciag

et al. Science 225: 932-935 (1984) and to reversed-phase HPLC columns by Thomas et al. Proc. Natl. Acad. Sci. USA 81: 357-361 (1984) as part of multistep purification protocols. Based, in part, on the relatively high abundance of r-aFGF in bacterial lysates, these two steps alone are herein demonstrated to be sufficient to obtain homogeneously pure r-aFGF of about 16,000 daltons as established by electrophoresis in polyacrylamide gels. These two steps alone do not yield pure aFGF from brain.

Mitogenic activity of the purified aFGF is determined by the incorporation of ^{3}H-thymidine into DNA by cell line fibroblasts, preferably BALB/c 3T3 A31 (American Type Culture Collection). The recombinant aFGF shows a peak response at about 1 ng protein or less per ml in the fibroblast stimulative assay.

Another embodiment of this invention is a method of promoting the healing of wounds by application of the novel peptide, either with or without heparin, preferably with heparin, about 1 to about 500 μg/cm^2 of this invention to the wound area either topically or subcutaneously in the wound in an amount of about 0.1 to 100 μg/cm^2 of surface for topical application.

For application, various pharmaceutical formulations are useful such as ointments, pastes, solutions, gels, solid water soluble polymers such as albumins, gelatins, hydroxypropyl cellulose, pluronics, tetronics or alginates in which the active ingredient is incorporated in amounts of about 1 to about 100 μg/ml.

The ability of aFGF to stimulate division in various cell types including fibroblasts, vascular and corneal endothelial cells and the like makes these peptides useful as pharmaceutical agents. These compounds can be used to treat wounds of mammals including humans by the administration of the novel r-aFGF to patients in need of such treatment.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Oligonucleotide Synthesis

Oligonucleotides were synthesized according to the technique described by Matteucci and Caruthers, J. Am. Chem. Soc. 103: 3185-3191 (1981); Beaucage and Caruthers, Tetrahedron Letters 22: 1859-1862 (1981). The base sequences of the synthesized oligonucleotides are shown in Table IV.

EXAMPLE 2

Assembly of the aFGF Gene

The oligonucleotides from Example 1 were assembled as two separate units, the N-terminal half (231 bp) and the C-terminal half (209 bp). The two halves were then combined for the intact synthetic gene, see Table III. Initially the oligonucleotides were kinased in the following reaction mixture: 70 mM Tris pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, 33 µM ATP, 0.3 units T4-polynucleotide kinase per µl, and 2.5 pmole oligonucleotide per µl. The mixture was incubated 1.5 hours at 37°C and then an additional hour after supplementing the mixture with 0.2

units/μl kinase and ATP to give a concentration of 100 mM. For radioactive labelling, the initial mixture contained 37 nCi/μl of [γ-^{32}P]-ATP.

The annealing and ligations were done in two separate reactions. In each reaction, 100 pmole of each of the eight oligonucleotides were added. In one reaction the oligonucleotides which make up one strand of the C-terminal or N-terminal half gene were kinased with the exception of the most 5' oligonucleotide. In the second reaction the oligonucleotides which make up the opposite strand were kinased, again with the exception of the most 5' oligonucleotide. Thus, in each reaction 3 oligonucleotides were kinased and 5 were not. When kinased oligonucleotides were used, 1 pmole of the ^{32}P-labelled oligonucleotide was also added for later identification of the products. Each reaction contained 200 μl with 70 mM Tris pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, and 30 μM ATP. The oligonucleotides were annealed by heating to 90°C for 4 minutes, then immediately transferring the reaction to 60°C and allowing it to cool slowly to 30°C. Ligation was done in 400 μl containing 60 mM Tris pH 7.6, 10 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, and 0.03 units T4 DNA ligase per μl by incubating at 20°C for 1.5 hours.

Polyacrylamide gel electrophoresis was used to purify the ligated oligonucleotides. The ligated oligonucleotides were precipitated with ethanol, redissolved in 20 μl of 80% formamide, 50 mM TRIS-borate pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol, and 0.1% (w/v) bromophenol blue. Each sample was heated at 90°C for 3 minutes and electrophoresed in a 10% urea-polyacrylamide gel at 75 watts for 5

hours. The oligonucleotide bands were visualized by exposing the gel to X-ray film.

The 231 base bands of each reaction for the N-terminus were cut out of the gel, combined, and eluted at 4°C in 1 ml of 0.5 M ammonium acetate, 1 mM EDTA pH 8. The eluted DNA was precipitated with ethanol and redissolved in 30 µl of 70 mM Tris pH 7.6, 5 mM DTT, and 10 mM $MgCl_2$. The 209 base bands of the C-terminus were eluted in the same manner.

The gel purified oligonucleotides were annealed prior to transformation by heating to 90°C for 4 minutes and slow cooling to 20°C. Assuming a 5% recovery from the initial starting oligonucleotides, 300 fmole and 100 fmole of recovered annealed 231 bp oligonucleotides were each ligated to 100 fmole of agarose gel purified 3.9 kb EcoRI-BamHI pBR322 fragment DNA in 20 µl of 25 mM Tris pH 7.8, 1 mM DTT, 10 mM $MgCl_2$, 0.4 mM ATP, with 1 unit T4 DNA ligase for 1 hour at 20°C. The annealed 209 bp oligonucleotides were ligated to agarose purified 3.9 kb BamHI-SalI pBR322 fragment DNA under the same conditions as the 231 base pair fragments. The ligation reactions were diluted 1:5 in H_2O and 1 µl of dilution was used to transform 20 µl of competent E. coli RR1 cells (BRL) as described by the supplier. The transformants were selected for growth in ampicillin and screened for the presence of the 231 bp EcoRI-BamHI or the 209 bp BamHI-SalI insert by restriction analysis of mini-lysate plasmid preparations.

The DNA sequence of clones containing the appropriate sized inserts was determined using the

chemical DNA sequence techniques of Maxam and Gilbert, Proc. Natl. Acad. Sci. USA 74: 560-564 (1977). Since none of the 231 bp clones had the correct sequence, a clone containing the correct sequence was prepared as follows. One clone with the correct sequence between the KpnI and BamHI sites was cleaved with KpnI and with SalI, which cleaves in the pBR322 vector. The 400 bp band was gel purified and ligated to the 3.8 kb KpnI-SalI band of a second clone containing the correct sequence from the EcoRI site to the KpnI site of the aFGF gene insert. After transformation, a resulting clone was sequenced to ensure the desired sequence had been obtained.

Since a clone containing the correct 209 bp sequence was obtained, no further manipulation of these clones was required. The final full-length aFGF synthetic gene was cloned by cleaving the N-terminal half clone with BamHI and SalI, treating with alkaline phosphatase, and ligating this to the gel purified 209 bp BamHI-SalI insert of the C-terminal half clone. This ligated material was used to transform competent RR1 cells as before.

EXAMPLE 3

Expression of the Synthetic Bovine aFGF Gene

The intact aFGF gene from Example 2 was incorporated into a modified pKK223-3 plasmid. The pKK223-3 plasmid (Pharmacia) contains the tac promoter which is a hybrid between regions of the trp promoter and the lac promoter, deBoer et al., Proc. Natl Acad. Sci. USA 80: 21-25 (1983). This plasmid also contains the rrnB rRNA transcription terminator,

a strong terminator sequence found to allow expression from strong promoters, Gentz et al., Proc. Natl. Acad. Sci. USA 78: 4936-4940 (1981); Brosius, Gene 27: 161-172 (1984). The pKK 223-3 plasmid was modified to remove the pBR322-derived SalI restriction enzyme site. This was accomplished by cleaving the pKK223-3 plasmid DNA with NdeI and NarI, and recircularizing the 2.7 kb DNA fragment to generate clone pKK2.7. The synthetic aFGF gene was then cleaved from its pBR322 vector and transferred to pKK2.7 after restricting this expression vector with EcoRI and SalI. This construction positions the initiating methionine of the synthetic gene 11 bases downstream of the Shine-Dalgarno ribosome binding site. The resulting recombinant, shown in Figure 1, was transformed into *E. coli* JM105 cells and also into *E. coli* DH5 cells.

The expression clones were grown at 37°C in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 0.4% glucose and 50 μg/ml ampicillin. When the optical density at 550 nm reached 0.5, IPTG was added to give 1 mM and growth was continued at 37°C for 3 hours. The cells were harvested by centrifugation at 10,000 x g for 20 minutes and the cells from 1 liter of culture were resuspended in 20 ml of 10mM sodium phosphate pH 7.2, (heparin-Sepharose buffer) 5 mM EDTA, 10N6 μg/ml TPCK, 34.3 μg/ml pepstatin A, 87 μg/ml PMSF, 15 μg/ml BPTI, and 34.3 μg/ml leupeptin. The resuspended cells were quickly frozen in a dry ice/ethanol bath and stored overnight at -70°C.

EXAMPLE 4

Extraction and Purification of Recombinant aFGF

The frozen cells from Example 3 were thawed, an additional 87 µg/ml PMSF was added, and the preparation was passed through a French pressure cell at 12,000 psi three times at 4°C. The resulting lysate was centrifuged at 93,000 x g for 30 minutes to remove cell debris. The supernatant was removed, adjusted to pH 7.2 with 1 M NaOH and loaded onto a 1.6 x 10 cm heparin-Sepharose (Pharmacia) column run at 4°C with a flow rate of 20 ml per hour collecting 2 ml fractions. The pellet was resuspended in 5 ml of 10 mM sodium phosphate, 2 M NaCl, pH 7.2, recentrifuged at 93,000 x g for 30 minutes and the supernatant diluted with three volumes of 10 mM sodium phosphate, pH 7.2, readjusted to pH 7.2 with 1 M NaOH, if necessary, and loaded onto the same heparin-Sepharose column. After loading, the column was washed with 10 mM sodium phosphate, 0.8 M NaCl, pH 7.2 until the absorbance at 280 nm fell to background. Bound r-aFGF was eluted as a single peak with 10 mM sodium phosphate, 1.5 M NaCl, pH 7.2. The pooled fractions from the heparin-Sepharose column were purified by reversed-phase HPLC using a 4.6 mm x 25 cm C_4 column (Separations Group) as described by Thomas et al., Proc. Natl. Acad. Sci. USA 81: 357-361 (1984). The r-aFGF eluted as a single major peak that was resolved from multiple minor contaminant peaks suggesting that the protein was homogeneously pure. Polyacrylamide gel electrophoresis was used to confirm purity. The purified r-aFGF was electrophoresed following the technique of

O'Farrell, J. Biol. Chem. 250: 4007-4021 (1975). Silver staining revealed a single band with a molecular mass of 16,000 daltons. Identity of the protein as aFGF was confirmed by both amino acid analysis and amino terminal sequence determination.

EXAMPLE 5

Biological Activity of Bovine Recombinant aFGF

Biological activity of the purified r-aFGF from Example 4 was evaluated using a fibroblast mitogenic assay as described by Thomas et al., J. Biol. Chem. 225: 5517-5520 (1980). BALB/c 3T3 A31 fibroblasts (American Type Culture Collection) were plated at 2 x 10^4 cells per 35 mm diameter well in culture media containing 10% heat-inactivated calf serum and incubated in 7% CO_2 (pH 7.35 ± 0.05). The cells became fully quiescent by replacing the media with 0.5% heat-inactivated calf serum 6 and again 24 hours later. At 55 hours after plating, 50 μg of heparin, test samples and 1.1 μg of dexamethasone were added, at 70 hours each well was supplemented with 2 μCi of [methyl-^{3}H]-thymidine (20 Ci/mmole, New England Nuclear) and 3 μg of unlabeled thymidine (Sigma), and at 95 hours the cells were processed for determination of radiolabel incorporated into DNA. Each dose-response point was the average of triplicate determinations. The results are shown in the following table:

TABLE VIII

Mitogenic Responses of BALB/c 3T3 Fibroblasts to Bovine r-aFGF

Concentration r-aFGF (ng/ml)	CPM r-aFGF	CPM Brain aFGF
0.003	268	231
0.010	498	329
0.031	1550	1017
0.100	7031	3684
0.316	9319	11353
1.000	4718	9050

The activity of the recombinant aFGF was equal to or slightly greater than that of brain derived aFGF. The purified r-aFGF had a half-maximal stimulation of DNA synthesis at about 71 pg/ml while purified brain derived aFGF had a half-maximal value 126 pg/ml.

EXAMPLE 6

Mutagenesis of the Bovine aFGF Gene to the Human aFGF Gene

To facilitate the mutagenesis of the bovine aFGF gene, the synthetic gene from Example 2 was transferred to M13mp19, a single-stranded DNA bacteriophage vector. Standard mutagenesis procedures were used as reported by Zoller and Smith, Methods in Enzymology, 100: 468-500 (1983); Norris et al., Nucleic Acids Research, 11: 5103-5112; and Zoller and Smith, DNA, 3: 479-488. The bovine pKK-aFGF plasmid

was cleaved with EcoRI and SalI, see Table III, and the resulting 440 bp fragment was agarose gel purified as in Example 2. Vector M13mp19 RF DNA (BRL) was cleaved with the same two endonucleases and the ends were subsequently dephosphorylated in 100 µl of 10 mM Tris pH 8.0 buffer with 100 units of bacterial alkaline phosphatase. A ligation was performed using 50 ng of the treated vector DNA and 12 ng of the aFGF gene fragment DNA in 10 µl of 25 mM Tris pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 0.4 mM ATP, with 2 units of T4 DNA ligase for 16 hours at 4°C. The reaction mixture was diluted 1:5 in H_2O and 1 µl of dilution was used to transform 20 µl of competent E. coli DH5 cells (BRL) as described by the supplier. The cells were plated with E. coli JM105 (Pharmacia) host cells in 0.03% X-gal and 0.3 mM IPTG; after incubation at 37°C colorless plaques were isolated. One phage clone containing the bovine aFGF gene was selected, M13mp19-aFGF.

Eight oligonucleotides were designed to specify the human sequence and synthesized, see Table VI.

Oligmer 8 contains an additional mutation in which thymine at site 386 in the bovine gene is replaced by cytosine in the human gene. This mutation allows the incorporation of a restriction site without altering the human aFGF amino acid sequence.

The human oligomers 1, 2, 3, 4, 6, and 8 were phosphorylated and 15 pmoles of each were annealed individually to 0.5 pmole of M13mp19-aFGF single-stranded phage DNA in 10 µl of 20 mM Tris pH

7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT for 10 minutes at 65°C followed by 10 minutes at 23°C. Closed-circular double-stranded molecules were then prepared in 20 µl of 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 25 mM NaCl, 5.5 mM DTT, 0.5 mM ATP, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, using 1 unit of T4 DNA ligase and 2 units of DNA polymerase I klenow fragment by incubation at 15°C for 17 hours. The preparations were each used to transform competent JM105 cells and the resulting transformant plaques were selected by hybridization with the appropriate oligomer which had been radio-labeled using ^{32}P-ATP and polynucleotide kinase. The conditions of hybridization were optimized for each probe to prevent formation of hybrids containing single base changes. Single-stranded DNA was isolated from the phage clone containing the human oligomer 4 mutations and the above procedure was repeated using the human oligomer 5 to generate a clone containing both the oligomer 4 and 5 mutations.

In the following procedures the bovine-to-human sequence mutations in these M13-based clones were combined into one pBR322-based clone. RF DNAs were prepared from clones containing the base changes specified by human oligomers 1, 2, 6, and 8. The DNA of the human 1 mutant clone was cleaved with EcoRI, the ends were dephosphorylated with bacterial alkaline phosphatase, and the DNA was cleaved with HindIII. The human 2 mutant DNA was cleaved with HindIII, treated with phosphatase, and then cleaved with BamHI. The human 6 mutant DNA was cleaved with BamHI, phosphatase treated, and subsequently cleaved

with ApaI. Likewise, the human 8 mutant DNA was cleaved with ApaI, the ends were dephosphorylated, and the DNA was cleaved with SalI. These four DNA preparations were electrophoresed through 2% agarose and the fragments of 45 bp, 190 bp, 135 bp, and 70 bp from the mutant DNAs containing human 1, 2, 6, and 8 mutations, respectively, were eluted from the gel. Approximately 60 fmoles of each fragment were collectively ligated to about 60 fmoles of a gel-purified 3.7 kb EcoRI-SalI fragment from pBR322 in 5 µl of 25 mM Tris pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 0.4 mM ATP, with 1.5 units of T4 DNA ligase for 16 hours at 12°C. The reaction mixture was diluted 1:5 in H_2O and 1 µl of dilution was used to transform 20 µl of competent E. coli DH5 cells (BRL) as described by the supplier. A clone containing the mutations specified by all four mutant oligomers was selected by hybridization with radiolabeled probes prepared from each of the oligomers. The 140 bp KpnI-BamHI DNA fragment isolated from cleaved RF DNA of the human 3 mutant M13 clone was ligated to endonuclease cleavage products of this human 1-2-6-8 mutant DNA and transformed into DH5 competent cells to generate a clone with the human 1-2-3-6-8 mutations. BamHI-PstI digestion fragments of this latter clone were ligated to the BamHI-PstI digestion fragments of RF DNA from the human 4-5 M13-based clone and the ligation mixture was used to transform DH5 competent cells. A clone containing the human 1-2-3-4-5-6-8 mutations was selected by oligomer hybridization and the aFGF gene EcoRI-SalI DNA fragment of this recombinant plasmid

was ligated to phosphatase-treated EcoRI-SalI-cleaved RF DNA of M13mp18 (BRL). Competent DH5 cells were transformed with this ligated DNA and the transformed cells were plated on JM105 host cells to generate an M13 clone. The single-stranded phage DNA of this clone was annealed with the human 7 oligomer and an M13 clone containing all the desired mutations was obtained following the procedure described above. RF DNA was prepared from this clone and cleaved with EcoRI and SalI. The resulting 440 bp band was gel purified and ligated to the 2.7 kb EcoRI-SalI DNA fragment of the pKK2.7 tac promoter expression vector. This DNA was used to transform competent DH5 cells thus generating the human pKK-aFGF expression clone used for production of the human form of aFGF.

The human r-aFGF was purified by the same procedure as that used for the bovine r-aFGF, see Example 4. The human r-aFGF was judged to be at least 99.75% pure based on the presence of a single intense band on a silver stained SDS electrophoretic gel loaded with 400 ng of purified human r-aFGF and having a sensitivity of about 1 ng/band. The protocol is described in Example 4.

The pure recombinant human aFGF was assayed for mitogenic activity using ^{3}H-thymidine incorporation into subconfluent BALB/c 3T3 cells as described for the bovine recombinant protein in Example 5. As previously observed with human brain-derived aFGF assayed on vascular endothelial cells, the recombinant human protein shows a greater difference in the heparin (50 μg/ml) activation than does either the brain-derived or recombinant

bovine aFGF, Gimenez-Gallego et al. Biochem. Biophys. Res. Comm. 135: 541-548(1986); the results of recombinant human aFGF on Balb/c 3T3 cells are shown in the following table:

TABLE IX

Mitogenic Responses of BALB/c 3T3 Fibroblasts to Human r-aFGF.

Concentration r-aFGF (picograms/ml)*	CPM	
	- heparin	+ heparin
0	3574	991
1	4156	1336
3.16	4216	1802
10.0	4092	2617
31.6	4155	4824
100	4274	10489
316	6060	14584
1000 (1 ng)	6811	10547
3160	7910	12357
10000	8597	9143
31600	9700	9057
100000	11166	9277
1000000 (1 μg)	15864	12425

*picogram = 10^{-12} grams

In the presence of heparin, the half-maximal stimulation occurs at about 42 pg/ml. In the absence of heparin the peak has not clearly been reached even at the highest concentration but must be greater than about 30 ng/ml.

WHAT IS CLAIMED IS:

1. Recombinant bovine acidic fibroblast growth factor.

2. Recombinant bovine acidic fibroblast growth factor having an amino acid sequence of:

1 10 20
PheAsnLeuProLeuGlyAsnTyrLysLysProLysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeu

30 40 50
ProAspGlyThrValAspGlyThrLysAspArgSerAspGlnHisIleGlnLeuGlnLeuCysAlaGluSerIleGlyGlu

60 70 80
ValTyrIleLysSerThrGluThrGlyGlnPheLeuAlaMetAspThrAspGlyLeuLeuTyrGlySerGlnThrProAsn

90 100
GluGluCysLeuPheLeuGluArgLeuGluGluAsnHisTyrAsnThrTyrIleSerLysLysHisAlaGluLysHisTrp

110 120 130
PheValGlyLeuLysLysAsnGlyArgSerLysLeuGlyProArgThrHisPheGlyGlnLysAlaIleLeuPheLeuPro

140
LeuProValSerSerAsp .

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3. The recombinant bovine acidic fibroblast growth factor of Claim 2 wherein there is attached to the phenylalanine at the first position a methionine.

4. Microheterogeneous forms of recombinant bovine acidic fibroblast growth factor.

5. A nucleotide sequence coding for bovine acidic fibroblast growth factor.

6. A nucleotide sequence coding for the recombinant bovine acidic fibroblast growth factor of Claim 2.

7. A nucleotide sequence coding for the recombinant bovine acidic fibroblast growth factor of Claim 3.

8. The nucleotide sequence of Claim 6 wherein the base sequence is any of the following:

```
TTQ AAQ CTN CCN CTN GGN AAQ TAQ AAP AAP CCN AAP CTN CTN TAQ TGQ TCN AAQ GGN GGN   60
            TTP     TTP                             TTP TTP         AGQ

TAQ TTQ CTN CGN ATQ CTN CCN GAQ GGN ACN GTN GAQ GGN ACN AAP GAQ CGN TCN GAQ CAP   120
        TTP AGP ATA TTP                                     AGP AGQ

CAQ ATQ CAP CTN CAP CTN TGQ GCN GAP TCN ATQ GGN GAP GTN TAQ ATQ AAP TCN ACN GAP   180
    ATA     TTP     TTP             AGQ ATA                 ATA     AGQ

ACN GGN CAP TTQ CTN GCN ATG GAQ ACN GAQ GGN CTN CTN TAQ GGN TCN CAP ACN CCN AAQ   240
            TTP                             TTP TTP         AGQ

GAP GAP TGQ CTN TTQ CTN GAP CGN CTN GAP GAP AAQ CAQ TAQ AAQ ACN TAQ ATQ TCN AAP   300
            TTP     TTP     AGP TTP                                 ATA AGQ

AAP CAQ GCN GAP AAP CAQ TGG TTQ GTN GGN CTN AAP AAP AAQ GGN CGN TCN AAP CTN GGN   360
                            TTP                             AGP AGQ     TTP

CCN CGN ACN CAQ TTQ GGN CAP AAP GCN ATQ CTN TTQ CTN CCN CTN CCN GTN TCN TCN GAQ:  420
    AGP                             ATA TTP     TTP     TTP         AGQ AGQ
```

where Q equals C or T, P equals A or G, and N equals A, T, C, or G.

9. The nucleotide sequence of Claim 8 wherein the code for phenylalanine is preceeded by a code for the methionine.

10. The nucleotide sequence of Claim 9 wherein the base sequence is:

```
1                  20                  40                  60                  80
AATTCATGTTCAATCTGCCACTGGGTAATTACAAAAAGCCAAAGCTTCTTTACTGCTCTAACGGTGGTTACTTTCTCCGC
    GTACAAGTTAGACGGTGACCCATTAATGTTTTTCGGTTTCGAAGAAATGACGAGATTGCCACCAATGAAAGAGGCG

                   100                 120                 140                 160
ATCCTGCCAGATGGTACCGTGGACGGCACCAAAGATCGTTCTGATCAACATATTCAACTGCAGCTGTGCGCCGAATCTAT
TAGGACGGTCTACCATGGCACCTGCCGTGGTTTCTAGCAAGACTAGTTGTATAAGTTGACGTCGACACGCGGCTTAGATA

                   180                 200                 220                 240
CGGTGAAGTTTACATCAAATCTACCGAAACTGGTCAATTCCTTGCCATGGACACTGATGGCCTGCTGTACGGATCCCAGA
GCCACTTCAAATGTAGTTTAGATGGCTTTGACCAGTTAAGGAACGGTACCTGTGACTACCGGACGACATGCCTAGGGTCT

                   260                 280                 300                 320
CCCCAAACGAGGAGTGCCTTTTCCTGGAGCGCCTGGAGGAAAACCATTACAACACCTACATCTCTAAAAAGCATGCTGAG
GGGGTTTGCTCCTCACGGAAAAGGACCTCGCGGACCTCCTTTTGGTAATGTTGTGGATGTAGAGATTTTTCGTACGACTC

                   340                 360                 380                 400
AAACATTGGTTCGTAGGCCTTAAGAAAAATGGCCGCTCTAAACTGGGCCCTCGTACTCACTTTGGTCAAAAAGCTATCCT
TTTGTAACCAAGCATCCGGAATTCTTTTTACCGGCGAGATTTGACCCGGGAGCATGAGTGAAACCAGTTTTTCGATAGGA

                   420                 440
GTTCCTGCCACTGCCAGTGAGCTCTGACTAATAGATATCG
CAAGGACGGTGACGGTCACTCGAGACTGATTATCTATAGCAGCT.
```

11. An expression plasmid comprising the nucleotide sequence of Claim 10 inserted therein.

12. The plasmid of Claim 11 wherein the structure is shown in Figure I.

13. The plasmid of Claim 11 wherein the plasmid is pBR322.

14. A host that is compatible with and contains the plasmid of Claim 11.

15. The host of Claim 14 wherein said host is _E. coli_.

16. The host of Claim 15 wherein said host is _E. coli_ JM105 or _E. coli_ DH5.

17. The plasmid of Claim 11 wherein said plasmid is capable of expressing the amino acid sequence of bovine acidic fibroblast growth factor.

18. A protein produced by the host of Claim 14 capable of stimulating DNA synthesis in responsive cells.

19. A process for the production of bovine acidic fibroblast growth factor comprising the following steps:

a. providing a plasmid comprising a nucleotide sequence coding for bovine acidic fibroblast growth factor, wherein the nucleotide sequence is capable of being expressed by a host containing the plasmid; followed by

b. incorporating the plasmid into the host; and

c. maintaining the host containing the plasmid under conditions suitable for expression of the nucleotide sequence producing bovine acidic fibroblast growth factor.

20. A process according to Claim 19, Step a, wherein the nucleotide sequence is that of Claim 10.

21. A process according to Claim 19, Step b, wherein the host is E. coli..

22. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the recombinant bovine acidic fibroblast growth factor of Claim 1.

23. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the recombinant bovine acidic fibroblast growth factor of Claim 2.

24. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the recombinant bovine acidic fibroblast growth factor of Claim 3.

25. A method of promoting wound healing which comprises the administration to a patient in need of such treatment of an effective wound healing amount of the recombinant bovine acidic fibroblast growth factor of Claim 2.

26. A method of promoting wound healing which comprises the administration to a patient in need of such treatment of an effective wound healing amount of the recombinant bovine acidic fibroblast growth factor of Claim 3.

27. Recombinant human acidic fibroblast growth factor.

28. Recombinant human acidic fibroblast growth factor having an amino acid sequence of:

```
 1                  10                            20
PheAsnLeuProProGlyAsnTyrLysLysProLysLeuLeuTyrCysSerAsnGlyGlyHisPheLeuArgIleLeu

      30                     40                        50
ProAspGlyThrValAspGlyThrArgAspArgSerAspGlnHisIleGlnLeuGlnLeuSerAlaGluSerValGlyGlu

              60                      70                            80
ValTyrIleLysSerThrGluThrGlyGlnTyrLeuAlaMetAspThrAspGlyLeuLeuTyrGlySerGlnThrProAsn

              90                               100
GluGluCysLeuPheLeuGluArgLeuGluGluAsnHisTyrAsnThrTyrIleSerLysLysHisAlaGluLysAsnTrp

      110                       120                            130
PheValGlyLeuLysLysAsnGlySerCysLysArgGlyProArgThrHisTyrGlyGlnLysAlaIleLeuPheLeuPro

            140
LeuProValSerSerAsp .
```

29. The recombinant human acidic fibroblast growth factor of Claim 28 wherein there is attached to the phenylalanine at the first position a methionine.

30. The recombinant human acidic fibroblast growth factor of Claim 28 wherein the phenylalanine at the first position is removed and the amino acid at the second position is either asparagine or asparatic acid.

31. Microheterogeneous forms of recombinant human acidic fibroblast growth factor.

```
1                   20                  40                  60                  80
AATTCATGTTCAATCTGCCACCGGGTAATTACAAAAAGCCAAAGCTTCTTTACTGCTCTAACGGTGGTCACTTTCTCCGC
    GTACAAGTTAGACGGTGGCCCATTAATGTTTTTCGGTTTCGAAGAAATGACGAGATTGCCACCAGTGAAAGAGGCG

                    100                 120                 140                 160
ATCCTGCCAGATGGTACCGTGGACGGCACCAGAGATCGTTCTGATCAACATATTCAACTGCAGCTGTCCGCCGAATCTGT
TAGGACGGTCTACCATGGCACCTGCCGTGGTCTCTAGCAAGACTAGTTGTATAAGTTGACGTCGACAGGCGGCTTAGACA

                    180                 200                 220                 240
CGGTGAAGTTTACATCAAATCTACCGAAACTGGTCAATACCTTGCCATGGACACTGATGGCCTGCTGTACGGATCCCAGA
GCCACTTCAAATGTAGTTTAGATGGCTTTGACCAGTTATGGAACGGTACCTGTGACTACCGGACGACATGCCTAGGGTCT

                    260                 280                 300                 320
CCCCAAACGAGGAGTGCCTTTTCCTGGAGCGCCTGGAGGAAAACCATTACAACACCTACATCTCTAAAAAGCATGCTGAG
GGGGTTTGCTCCTCACGGAAAAGGACCTCGCGGACCTCCTTTTGGTAATGTTGTGGATGTAGAGATTTTTCGTACGACTC

                    340                 360                 380                 400
AAAAATTGGTTCGTAGGCCTTAAGAAAAATGGCAGCTGTAAACGCGGCCCTCGTACTCACTATGGCCAAAAAGCTATCCT
TTTTTAACCAAGCATCCGGAATTCTTTTTACCGTCGACATTTGCGCCGGGAGCATGAGTGATACCGGTTTTTCGATAGGA

                    420                 440
GTTCCTGCCACTGCCAGTGAGCTCTGACTAATAGATATCG
CAAGGACGGTGACGGTCACTCGAGACTGATTATCTATAGCAGCT.
```

35. The nucleotide sequence of Claim 10 wherein the base sequence is substituted by point mutations to give the base sequence of Claim 34.

32. A nucleotide sequence coding for human recombinant acidic fibroblast growth factor.

33. A nucleotide sequence coding for the human recombinant acidic fibroblast growth factor of Claim 29.

34. The nucleotide sequence of Claim 33 wherein the base sequence is:

a. providing a plasmid comprising a nucleotide sequence coding for human aFGF, wherein the nucleotide sequence is capable of being expressed by a host containing the plasmid; followed by

b. incorporating the plasmid into the host; and

c. maintaining the host containing the plasmid under conditions suitable for expression of the nucleotide sequence producing human aFGF.

46. A process according to Claim 45, Step a, wherein the nucleotide sequence is that of Claim 34.

47. A process according to Claim 45, step b, wherein the host is E. coli.

48. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the recombinant human acidic fibroblast growth factor of Claim 27.

49. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the recombinant human acidic fibroblast growth factor of Claim 28.

36. An expression plasmid comprising the nucleotide sequence of Claim 34 inserted therein.

37. The plasmid of Claim 36 wherein the structure is shown in Figure I.

38. The plasmid of Claim 37 wherein the plasmid is pBR322.

39. A host that is compatible with and contains the plasmid of Claim 36.

40. A host of Claim 39 which is E. coli.

41. The host of Claim 40 wherein said host is E. coli JM105 or E. coli DH5.

42. The plasmid of Claim 36 which is capable of expressing the gene for human acidic fibroblast growth factor.

43. The plasmid of Claim 36 which is capable of expressing the synthetic nucleotide sequence for human acidic fibroblast growth factor.

44. A protein produced by the host of Claim 39 capable of stimulating DNA synthesis in responsive cells.

45. A process for the production of human aFGF comprising the following steps:

50. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amound of the recombinant human acidic fibroblast growth factor of Claim 29.

51. A wound healing pharmaceutical composition comprising a pharmaceutical carrier and an effective wound healing amount of the microheterogeneous forms of recombinant human acidic fibroblast growth factor.

52. A method of promoting wound healing which comprises the administration to a patient in need of such treatment of an effective wound healing amount of the recombinant human acidic fibroblast growth factor of Claim 27.

53. A method of promoting wound healing which comprises the administration to a patient in need of such treatment of an effective wound healing amount of the recombinant human acidic fibroblast growth factor of Claim 28.

54. A method of promoting wound healing which comprises the administration to a patient in need of such treatment of an effective wound healing amount of the recombinant human acidic fibroblast growth factor of Claim 29.

55. A method of promoting wound healing which comprises the administration to a patient in

need of such treatment of an effective growth promoting amount of the microheterogeneous forms of recombinant human acidic fibroblast growth factor.

56. A method of purifying recombinant acidic fibroblast growth factor (aFGF) in pure form comprising the following steps:

a. Partial purification of recombinant aFGF by an affinity chromatography matrix and an acceptable eluant; followed by

b. final purification of partially purified recombinant aFGF by reverse phase high performance liquid chromatography using an alkyl silane substrate and an acceptable eluant.

57. A method according to Claim 56, Step a, wherein the affinity matrix is heparin-Sepharose.

58. A method according to Claim 56, Step b, wherein the alkyl silane substrate contains between 3 and 18 carbon atoms.

59. A method according to Claim 56, Step b, wherein the alkyl silane substrate contains 4 carbon atoms.

60. A method according to Claim 56, Step a, wherein aFGF is eluted with sodium chloride.

61. A method according to Claim 56, Step b, wherein aFGF is purified by an elution gradient consisting of an acid and an organic solvent.

62. A method according to Claim 61 wherein the acid is trifluoroacetic acid, phosphoric acid or acetic acid.

63. A method according to Claim 61 wherein the organic solvent is acetonitrile or ethanol.

Neu eingereicht / Newly filed
No

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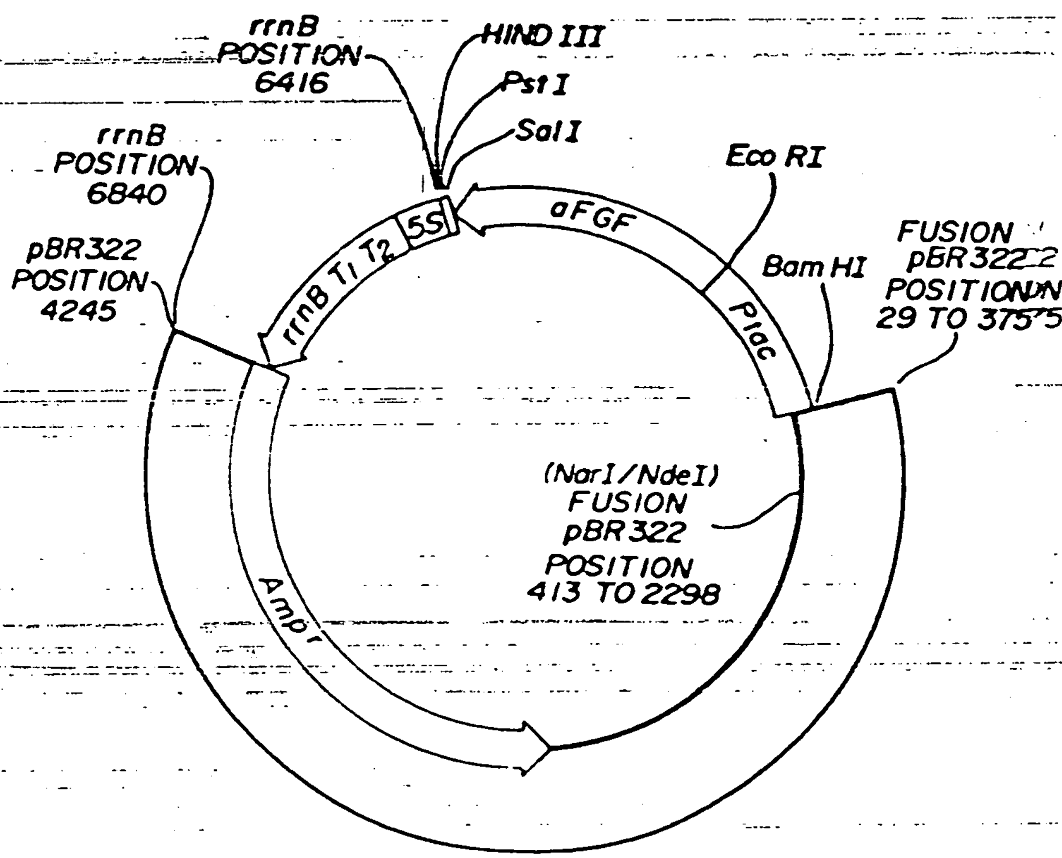
FIG-1



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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number: EP 87 30 6066

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	BIOLOGICAL ABSTRACTS/RRM, vol. 31, ref.no. 46304, 1986, Philadelphia, Pa., US J.C. FIDDES et al.: "Isolation and characterization of clones encoding basic and acidic fibroblast growth factors." & J. CELL BIOCHEM, 1986, vol. (, no. 10, part C, page 149 * Title *	27,32	C 12 N 15/00 A 61 K 37/02 C 07 K 13/00 C 07 H 21/04

Y	CHEMICAL ABSTRACTS, vol. 104, no. 21, May 26, 1986, page 118, ref.no. 180694b; Columbus, Ohio, US G. GIMENEZ-GALLEGO et al.: "Human brain-derived acidic and basic fibroblast growth factors: amino terminal sequences and specific mitogenic activities." & BIOCHEM. BIOPHYS. RES. COMMUN., 1986, vol. 135, no. 2, pages 541-548		
	./.		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
A 61 K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely: [illegible],27-34,36,39,42,44,45,48-51, 56

Claims searched incompletely:

Claims not searched: 52-55

Reason for the limitation of the search:

Method for treatment of the human or animal body by surgery or therapy (see art. 52(4) of the European Patent Convention).

Place of search	Date of completion of the search	Examiner
THE HAGUE	[illegible]-11-1987	YEATS

CATEGORY OF CITED DOCUMENTS

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons
& : member of the same patent family, corresponding document

1503

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☒ CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.

☒ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid,

namely claims: 11,22,27-34,36,39,42,44,45,48-56

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

☐ LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions,

namely:

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid,

namely claims:

☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims,

namely claims:

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European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application number
EP 87 30 6066
- 2 -

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
	* Abstract *	1,2,5, 27,28, 32	

Y,D	SCIENCE, vol. 230, no. 4732, 1985, pages 1385-1388; USA G. GIMENEZ-GALLEGO et al.: "Brain-derived acidic fibroblast growth factor: Complete amino acid sequence and homologies."		
	* Abstract; page 1385, column 1, line 1 - page 1386, column 2, line 31; figure 1 *	1,2,5, 27,28, 32,48, 56	

Y	EP-A-0 131 150 (MERCK & CO. INC.)		
	* Abstract; exemple, step 5; claims *	1,48, 56	TECHNICAL FIELDS SEARCHED (Int. Cl 4)

P,Y	CHEMICAL ABSTRACTS, vol. 105, no. 19, November 10, 1985, page 89, ref.no. 165184n; Columbus, Ohio, US G. GIMENEZ-GALLEGO et al.: "The complete amino acid sequence of human brain-derived acidic fibroblast growth factor." & BIOCHEM. BIOPHYS. RES. COMMUN, 1986, vol. 138, no. 2, pages 611-617 (Cat. D)		
	* Abstract *	27,28, 32	

P,X	BIOLOGICAL ABSTRACTS/RRM, vol. 33, 1987, ref. no. 40030; Philadelphia, Pa., US ./.		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner

CATEGORY OF CITED DOCUMENTS

X particularly relevant if taken alone
Y particularly relevant if combined with another document of the same category
A technological background
O non-written disclosure
P intermediate document
T theory or principle underlying the invention
E earlier patent document, but published on, or after the filing date
D document cited in the application
L document cited for other reasons
& member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application number
EP 87 30 6066

- 3 -

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
	J. ABRAHAM et al.: "Cloning and characterization of the genes for the angiogenic proteins basic and acidic fibroblast growth factor." & J. CELL BIOCHEMSUPPL., 1987, vol. 0, no. 11, part A, page 50 * Title *	27,28, 32	
T	BIO/TECHNOLOGY, vol. 5, no. 9, September 1987, pages 960-965; London, GB D.L. LINEMEYER et al.: "Expression in Escherichia coli of a chemically synthesized gene for biologically active bovine acidic fibroblast growth factor." * Whole article *	1-11, 56	TECHNICAL FIELDS SEARCHED (Int. Cl 4)
P,X	SCIENCE, vol. 233, no. 4763, August 1, 1986, pages 541-545; USA M. JAYE et al.: "Human endothelial cell growth factor: Cloning, nucleotide sequence, and chromosome localization." * Abstract; page 541, column 1, line 1 - column 2, line 29; page 542, column 1, line 24 - column 2, line 13; figures 2,3 *	32-34	
E	EP-A-0 225 701 (TAKEDE CHEM. IND. LTD) * Page 7, line 1 - page 8, line 12; claims *	27,32, 45	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner

CATEGORY OF CITED DOCUMENTS

X particularly relevant if taken alone
Y particularly relevant if combined with another document of the same category
A technological background
O non-written disclosure
P intermediate document

T theory or principle underlying the invention
E earlier patent document, but published on, or after the filing date
D document cited in the application
L document cited for other reasons
& member of the same patent family, corresponding document

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